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Predicting the Concentration of Verotoxin-Producing *Escherichia coli* Bacteria during Processing and Storage of Fermented Raw-Meat Sausages

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A model to predict the population density of verotoxigenic *Escherichia coli* (VTEC) throughout the elaboration and storage of fermented raw-meat sausages (FRMS) was developed. Probabilistic and kinetic measurement data sets collected from publicly available resources were completed with new measurements when required and used to quantify the dependence of VTEC growth and inactivation on the temperature, pH, water activity (a_w), and concentration of lactic acid. Predictions were compared with observations in VTEC-contaminated FRMS manufactured in a pilot plant. Slight differences in the reduction of VTEC were predicted according to the fermentation temperature, 24 or 34°C, with greater inactivation at the highest temperature. The greatest reduction was observed during storage at high temperatures. A population decrease greater than 6 decimal logarithmic units was observed after 66 days of storage at 25°C, while a reduction of only ca. 1 logarithmic unit was detected at 12°C. The performance of our model and other modeling approaches was evaluated throughout the processing of dry and semidry FRMS. The greatest inactivation of VTEC was predicted in dry FRMS with long drying periods, while the smallest reduction was predicted in semidry FMRS with short drying periods. The model is implemented in a computing tool, *E. coli* SafeFerment (EcSF), freely available from http://www.ifr.ac.uk/safety/EcoliSafeFerment. EcSF integrates growth, probability of growth, and thermal and nonthermal inactivation models to predict the VTEC concentration throughout FRMS manufacturing and storage under constant or fluctuating environmental conditions.

Verotoxin-producing *Escherichia coli* (VTEC) organisms, including serotype O157:H7, are food-borne pathogens causing severe illness in humans (1). A subgroup of VTEC capable of causing hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) constitutes the enterohemorrhagic *E. coli* (EHEC). From those, a range of serogroups (i.e., O157, O26, O103, O91, O145, and O111) are very frequently associated with public health issues (2).

Cattle are the principal reservoir of VTEC (3, 4). Transmission mainly occurs through consumption of undercooked infected beef, unpasteurized dairy products, vegetables, or contaminated water (3). Person-to-person transmission has also been reported (5). The dose associated with food-borne *E. coli* O157:H7 outbreaks has been estimated to be between 2 and 2,000 bacteria. Such a low dose means that this microorganism does not need to grow in a product to be able to cause illness (6). In the United States, VTEC is responsible for an estimated 260,000 illnesses annually, with 3,700 annual hospitalizations and 20 annual deaths (7). In Europe, the estimated number of cases per year is approximately 3,000 to 4,000 (5).

Fermented raw-meat sausages (FRMS) are products in which a combination of fermentation, drying, spices, salt, sodium nitrite, and the starter culture activity, generating lactic acid and reducing the medium pH, inhibits the growth and/or reduces the survival of pathogenic bacteria. Several food-borne outbreaks involving FRMS contaminated with VTEC have been reported (8, 9). In the European Union, minced and/or fermented meat and products thereof are considered a hazard to public health because of the possibility of VTEC contamination (10). In the United States, a reduction of 5 logarithmic units in the population of *E. coli* during the manufacturing of FRMS is required (11). In Canada, a 5-logarithmic-unit reduction is recommended, while in Australia, the required reduction is 3 logarithmic units (12).

The increasing number of food-borne outbreaks caused by VTEC and the severity of VTEC infections are the reasons behind the intense efforts to understand its population dynamics regarding growth and decay rates and also growth limits under several environmental conditions (13, 14). Models to predict the population kinetics of *E. coli* and other bacteria in foods are available at several public resources, such as the ComBase portal (15), the PMP (16), or FoodRisk.org (17). The dependence of the growth of *E. coli* on the environment has been modeled under a wide range of food conditions (18–24). Growth/no-growth boundaries (13, 22) and inactivation of *E. coli* (25–30) have also been estimated in broth and foods including FMRS. Specifically, the inactivation of

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E. coli O157:H7 has been modeled as a function of changes in the medium due to the metabolic activity of the starter culture and yeast during fermentation in olives (31) and as a function of pH and water activity (a_w) in soudjouk-style fermented sausages during the process of fermentation and drying (32). The publically available software THERM predicts the growth or lack of growth of *E. coli* O157:H7 and other pathogens as a function of the time-temperature history of raw-meat products (33); more specifically, tools implementing models to predict the inactivation of *E. coli* O157:H7 in raw meat (19) and in fermented meat products (34, 35) are available from the Australia Food Safety Center of Excellence. However, predicting the response of VTEC throughout the range of conditions that comprise growth and no-growth environments characteristic to FRMS manufacturing and storage has not been approached yet.

The aims of this work were to quantify the response of VTEC under FRMS manufacturing and storage conditions and to develop a user-friendly dynamic modeling framework to predict the concentration of VTEC organisms throughout those processes. We carried out an extensive search in the literature and publicly available data sources regarding growth and inactivation rates and growth/no-growth limits under different conditions of temperature, pH, a_w, and lactic acid in laboratory media and food products. In addition, new data were experimentally generated to address data gaps and FRMS were manufactured in a pilot plant to evaluate the predictions of the model of the population density of VTEC during manufacturing and storage.

With the aim of facilitating the application of the model, it has been implemented in an Excel add-in called *E. coli* SafeFerment (EcSF), which is freely available at http://www.ifr.ac.uk/safety/EcoliSafeFerment. The EcSF tool combines growth, probability of growth, and inactivation models to give predictions of VTEC concentration at any stage of the production and storage of FRMS under constant or fluctuating environmental conditions.

MATERIALS AND METHODS

Data collection. The data sets used for developing the models for VTEC were obtained from the ComBase database (15) or from the literature (13, 20, 22, 35–38) or were kindly provided by the Food Safety Centre, University of Tasmania (39, 40). Additional data were generated in this work to address data gaps (see Table S1 in the supplemental material). Table S2 in the supplemental material shows the serotypes and strains used for publicly available data and for newly generated data.

Data generation. (i) Strains and inoculum and medium preparation. Two verotoxigenic *E. coli* strains were used in this work to measure nonthermal inactivation rates as well as to generate growth/no growth observations at various combinations of temperature, pH, and a_w and in the presence of several concentrations of lactic acid. These were the O103:H25 strain implicated in an outbreak caused by fermented sausages containing mutton beef (morr) in 2006 in Norway (41) and the O157:H7 218 strain implicated in an outbreak caused by cold-smoked fermented sausages in Sweden in 2002 (42).

Rifampin-resistant (Rif^r) strains *E. coli* O157:H7 218Rif and O103:H25 Rif^r, previously constructed and evaluated for growth and inactivation in laboratory media under various environmental conditions, were used to inoculate FRMS (28, 30). Growth of wild-type and Rif^r mutant strains was evaluated in tryptone soy broth (TSB; Oxoid, United Kingdom) and in TSB with 10,000 ppm of lactic acid at pH 5.5; inactivation was measured in TSB with 45,000 ppm of lactic acid at pH 4.0 and an a_w value equal to 0.90. The growth and inactivation responses of Rif^r strains were identical to those of the wild-type strains under all tested conditions (data not shown).

Strain cultures were stored at −70°C with 20% glycerol. Inocula of wild-type strains were subcultured successively three times in TSB at 37°C for 24 h, immediately prior to the experiments. Rif^r strain inocula were prepared as previously described (28). When required, the medium pH was adjusted aseptically with 6 N NaOH and 1 N HCl, and lactic acid (90.08 g/liter; 90% acid) and NaCl were added to the media prior to autoclaving. Water activity was based on NaCl supplementation of TSB, already containing 0.5%, and it was estimated according to the equation $a_w = -0.0071 \times \text{percent NaCl} + 1.0054$ as previously reported (43).

(ii) Nonthermal inactivation experiments. A new data set of 96 nonthermal inactivation curves, in which a_w was the inhibitory factor, was generated at all possible combinations of several temperatures (12, 16, 21, and 25°C), pH values (4, 5, and 6), a_w values (0.90 and 0.94), and lactic acid concentrations (5,000, 10,000, 20,000, and 45,000 ppm). TSB was conditioned according to the experimental design and maintained at the target temperature prior to the inoculation of approximately 10^7 CFU/ml of *E. coli* O103:H25 or *E. coli* O157:H7. At adequate time intervals, samples were plated out on tryptone soy agar (TSA; Oxoid); plates were incubated at 37°C for 48 h. The reason for this extended period of incubation instead of the traditional 24 h is that 37°C may not be an optimum temperature for the recovery of stressed bacteria and for subsequent colony formation. Despite this precaution, the absolute number of colony counts is likely to be affected. However, the recovery environment is assumed to have the same impact on the absolute counts at all sampling times during the inactivation process, so the estimation of the population inactivation rate should not be significantly affected by the recovery conditions. However, this is not experimentally proven and could be one of the reasons behind the complexity and lack of linearity reported for bacterial inactivation.

(iii) Growth/no-growth experiments. Two new data sets were generated. The first set was generated to evaluate the ability to grow under 288 conditions resulting from combining several temperatures (10, 16, and 20°C), pH values (4.8, 5.1, 5.4, 5.7, 6.0, and 6.3), a_ws (0.94, 0.95, 0.96, and 0.97), and lactic acid concentrations (5,000, 10,000, 20,000, and 45,000 ppm). TSB was conditioned and set to the target temperature. Volumes of 225 μl were dispensed into Bioscreen microplates and inoculated with 25 μl of 100-fold dilutions of *E. coli* O103:H25. In the second set, the ability of *E. coli* to grow was evaluated under 144 conditions resulting from combining a wider range of temperatures (10, 16, 20, and 25°C) and higher a_ws (0.96, and 0.99) with the same pH values and lactic acid concentrations as for the first data set. TSB was prepared as described above and inoculated with 25 μl of a mixture of equal parts of a 100-fold-diluted culture of *E. coli* O103:H25 and a 100-fold-diluted culture of *E. coli* O157:H7 218.

The borders of the plates were filled with 250 μl of sterile TSB or with 225 μl of TSB inoculated with 25 μl of 100-fold-diluted *E. coli* to follow normal growth in TSB. Optical density measurements were performed at 600 nm (Bioscreen C analyzer) upon inoculation and then daily for up to 33 days. Microplates were stored in plastic boxes to avoid liquid evaporation and incubated at the target temperatures. At least two replicates were recorded for each strain and combination of environmental conditions.

(iv) Manufacturing of FRMS in a pilot plant. Sausage production was carried out in a biosafety level 3 laboratory as previously described (28). The "Norwegian sausage" recipe used was as follows (g per kg): beef, 378.5; pork, 378.5; pork back fat, 200; glucose, 40; sodium nitrite, 0.08; ascorbic acid, 0.5; and sausage spices (commercial premix), 3. LS-25 starter culture (Gewürzmüller, Germany) was added according to the manufacturer's instructions. After chopping and mixing, approximately 10^7 CFU/g of *E. coli* O157:H7 218Rif was added together with the starter culture with gentle mixing before stuffing in fibrous casings (diameter, 70 mm) to sausages of approximately 500 to 700 g. To cover all samplings (1 sausage per sampling), at least 10 sausages were made for each batch. The temperature of the batter at stuffing was −2 to 0°C. The sausages were conditioned at ambient temperature and humidity for approximately 2 h and then transferred to the climate chamber. The temperature and relative

TABLE 1 Observed and predicted reductions in the concentration of VTEC during FRMS elaboration and storage and discrepancy and bias percentages between observed and predicted concentrations throughout the process

Food	Fermentation temp in °C (no. of days)	Maturation temp in °C (no. of days)	Storage temp in °C (no. of days)	Observed and predicted reductions in VTEC (log_{10} CFU/g)			No. of data points	Discrepancy (%)	Bias (%)
				Fermentation	Maturation	Storage			
Norwegian sausage	24 (3) + 19 (3)	17 (4) + 14 (17)		0.41, 0.26	0.79, 0.14		8	5.19	1.35
Swedish mettwurst	22 (4) + 21 (2)	16 (4)		0.55, 0.28	0.28, 0.20		6	6.82	1.72
Norwegian sausage	24 (3) + 19 (3)	17 (7) + 14 (7)	12 (66)	0.59, 0.35	0.44, 0.51	1.19, 1.69	7	3.98	0.00449
	24 (3) + 19 (3)	17 (7) + 14 (7)	16 (66)	0.59, 0.35	0.44, 0.51	1.34, 2.49	7	10.53	−3.39
	24 (3) + 19 (3)	17 (7) + 14 (7)	21 (66)	0.59, 0.35	0.44, 0.51	4.30, 3.92	7	11.81	3.12
	24 (3) + 19 (3)	17 (7) + 14 (7)	25 (66)	0.59, 0.35	0.44, 0.51	>6.00, 5.58	7	13.49	6.59
Norwegian sausage	34 (3) + 19 (3)	17 (7) + 14 (7)	12 (66)	0.59, 0.55	0.26, 0.55	0.77, 1.61	7	7.90	−2.02
	34 (3) + 19 (3)	17 (7) + 14 (7)	16 (66)	0.59, 0.55	0.26, 0.55	1.80, 2.34	7	6.64	−1.57
	34 (3) + 19 (3)	17 (7) + 14 (7)	21 (66)	0.59, 0.55	0.26, 0.55	3.34, 3.74	7	11.86	−5.10
	34 (3) + 19 (3)	17 (7) + 14 (7)	25 (66)	0.59, 0.55	0.26, 0.55	>5.94, 5.20	7	16.33	2.95

humidity (RH) regimen in the climate chamber was as follows: 3 days at 24°C and 94% RH, 3 days at 18°C and 85% RH, 4 days at 17°C and 81% RH, and 17 days at 15°C and 75% RH (Table 1). The internal sausage temperature was monitored by an automatic temperature logging device (Termometerfabriken Viking AB, Eskilstuna, Sweden). For the four periods of the regimen described above, the actual internal temperatures were, on average, 24.2°C, 18.6°C, 17.0°C, and 14.2°C, respectively, with deviations of ±0.4°C.

Meat batter representing a typical "Swedish mettwurst"-type sausage was delivered frozen in portions directly from a Swedish sausage factory (exact recipe not provided).

E. coli O157:H7 218Rif and starter culture (FSC 111; Chr. Hansen A/S, Denmark) were mixed into the batter before stuffing and weighing, as described above. The temperature and RH regimen in the chamber was 24 h at 19 to 21°C and 40 to 50% RH, 3 days at 22°C and 70% RH, 2 days at 20°C and 60% RH, and 4 days at 16°C and 60% RH (Table 1). The actual internal temperatures were, on average, 20°C, 22.4°C, 20.7°C, and 15.7°C ± 0.4°C, respectively. The finished sausages were vacuum packed and stored in the dark at 4°C.

To study the effect of the fermentation temperature and of storage conditions, Norwegian sausages were inoculated with ca. 10^7 CFU/g of an equal-part mixture of *E. coli* O157:H7 218Rif and *E. coli* O103:H25 Rif^r. The temperature regimen was 3 days at 24°C or 34°C, 3 days at 18°C, 7 days at 17°C, and 7 days at 15°C (Table 1). The finished product was vacuum packed and stored at different temperatures (12, 16, 21, or 25°C) for up to 66 days (Table 1).

At appropriate sampling times, an entire sausage was removed; 10 g of the sausage middle section was mixed in a stomacher with 90 ml of 0.4% peptone water. Water activity was analyzed using the Aqualab instrument (Decagon Devices, Pullman, WA) according to the manufacturer's instructions. The pH was measured on the water phase in the stomacher mixing. The concentration of lactic acid was determined by a commercial analytical laboratory (Eurofins Norge, Moss, Norway). Samples were plated out on TSA plates (Oxoid) containing 100 μg/ml of rifampin (Sigma-Aldrich, Norway).

(v) Parameter estimation. When required, the maximum specific growth rate was estimated by fitting the model of Baranyi and Roberts (44) to growth curves, while thermal and nonthermal specific inactivation rates were estimated from the slope of the linear part of the inactivation curve expressed as the natural logarithm of the concentration versus time.

(vi) Model development. The bacterial concentration, x, at any time, t, in fluctuating environmental conditions was modeled as follows:

$$\frac{dx(t)}{dt} = r(t)u(t)x(t) \tag{1}$$

where $u(t)$ describes the transition from the exponential growth phase to the stationary phase.

$$u(t) = 1 - \left[\frac{x(t)}{x_{max}}\right] \tag{2}$$

The maximum bacterial concentration, x_{max}, is taken to be constant and equal to 10^9 CFU per ml or gram.

$$r(t) = \begin{cases} \mu_{max}(t) & \text{if } P(t) > 0.5 \text{ and temp} < 47°C \\ \varphi(t) & \text{if } P(t) < 0.5 \text{ and temp} < 47°C \\ \tau(t) & \text{if temp} \geq 47°C \end{cases} \tag{3}$$

$\mu_{max}(t)$ describes the maximum specific growth rate, $\varphi(t)$ the nonthermal specific inactivation rate, $\tau(t)$ the thermal specific inactivation rate, and $P(t)$ the probability of growth according to the environmental conditions at a given time, t.

The dependence of μ_{max} on the temperature, pH, a_w, and lactic acid concentration was modeled with a second-order polynomial:

$$\ln(\mu_{max}) = a_0 + a_1T + a_2\text{pH} + a_3b_w + a_{12}T\text{pH} + a_{13}T\text{b}_w + a_{23}\text{pH}b_w + a_{11}T^2 + a_{22}\text{pH}^2 + a_{33}\text{b}_w^2 + a_4\text{UL} \tag{4}$$

where a_i represents the model parameters, T is the temperature in Celsius scale (°C), $b_w = \sqrt{1 - a_w}$ (45), and UL is the concentration of undissociated lactic acid (mM) which was estimated according to the Henderson-Hasselbalch equation as $UL = L/(1 + 10^{pH - pK_a})$, where L is the total amount of lactic acid (mM) produced during fermentation and the pK_a is the pH value at which the undissociated and dissociated forms of the acid are balanced; this value is 3.86 for lactic acid. Parameters in equation 4 were fitted in two linear regression steps as previously described (46). In the first step the a_0 coefficient as and all the coefficients accounting for the effects of the temperature, pH, and a_w were fitted to the data described in Table S1 in the supplemental material in order to obtain the three-factor core model. The differences between the predictions of this model and the observed rates in the presence of lactic acid (see Table S1 in the supplemental material) were used to estimate the a_4 coefficient and extend the model with the fourth environmental factor, UL. All environmental factors exerted a significant effect on the growth rate.

The relationship between $\varphi(t)$, i.e., the nonthermal specific inactivation rate, and the temperature, pH, a_w, and lactic acid was modeled by an Arrhenius-type function as previously reported (23):

$$\ln(\varphi) = b_0 + b_1\left(\frac{1}{\text{TK}}\right) + b_2\left(\frac{1}{\text{pH}}\right) + b_3\left(\frac{1}{\text{b}_w}\right) + b_4\text{UL} + F \tag{5}$$

where b_i and F are model parameters and TK is the absolute temperature

in Kelvin scale. As previously described (46), the b_0, b_1, b_2, and b_3 parameters of the core model were estimated by linear regression in a first step from data described in Table S1 in the supplemental material. *F* tests (47) were carried out to evaluate if the effect of each environmental variable on the inactivation rate was significant. The apparent activation energy, E_a, a typical parameter of the Arrhenius model, can be estimated as $E_a = b_1/K$, where *K* is the universal gas constant equal to ca. 8.314 J K^{-1} mol^{-1}. In a second linear regression step, the core model was extended with the effect of the lactic acid concentration by estimating the b_4 coefficient from the differences between model predictions and the observed rates in the presence of lactic acid (see Table S1 in the supplemental material). The parameter *F* was a factor estimated from the bias between model predictions and rates measured with the most resistant strain studied in this work.

The dependence of τ, i.e., the thermal specific inactivation rate at temperatures greater than 47°C, on the temperature was modeled as follows:

$$\ln(\tau) = c_0 + c_1 T \tag{6}$$

c_0 and c_1 were estimated by linear regression.

$P(t)$ expresses the relationship between the probability of growth and the environmental conditions. This was estimated according to a logistic regression model as previously described (13):

$$\begin{aligned}\ln\left(\frac{P}{1-P}\right) = {} & d_0 + d_1\ln(T - T_{\min}) + d_2\ln(\mathrm{pH} - \mathrm{pH}_{\min}) \\ & + d_3\ln(a_w - a_{w\min}) + d_{12}\ln(T - T_{\min})\ln(\mathrm{pH} - \mathrm{pH}_{\min}) \\ & + d_{13}\ln(T - T_{\min})\ln(a_w - a_{w\min}) \\ & + d_{23}\ln(\mathrm{pH} - \mathrm{pH}_{\min})\ln(a_w - a_{w\min}) \\ & + d_4\ln\left[1 - \left(\frac{\mathrm{UL}}{\mathrm{UL}_{\mathrm{MIC}}}\right)\right]\end{aligned} \tag{7}$$

where d_i represents the model coefficients estimated by logistic regression and $T_{\min}$, $\mathrm{pH}_{\min}$, $a_{w\min}$, and $\mathrm{UL}_{\mathrm{MIC}}$ are the limiting values of the temperature (°C), pH, a_w, and undissociated lactic acid (mM), respectively, that prevent growth of *E. coli*. $T_{\min}$, $\mathrm{pH}_{\min}$, and $a_{w\min}$ were fixed to 7, 3.5, and 0.94, respectively, as estimated from ComBase, while $\mathrm{UL}_{\mathrm{MIC}}$ was fixed to 25 as previously reported (48). The core model coefficients for the temperature, pH, and a_w were estimated by linear logistic regression using SAS 9.3 (49) with backwards parameter selection in order to omit unnecessary coefficients from the model. The core model was extended with the effect of lactic acid by estimating the value of d_4 that maximizes the likelihood between model predictions and growth/no-growth observations with lactic acid (see Table S1 in the supplemental material).

Computing tool development. A forward Euler method (50) has been implemented in Visual basic code to simulate equation 1 under fluctuating conditions of temperature, pH, a_w, and lactic acid. The program is published as an Excel add-in named *E. coli* SafeFerment (EcSF) freely available at http://www.ifr.ac.uk/safety/EcoliSafeFerment.

Model performance indices. Bias and discrepancy percentages between model predictions (pred) and observations (obs) were estimated as previously reported (51):

$$\text{Discrepancy}\,(\%) = (e^A - 1)100 \tag{8}$$

where

$$A = \sqrt{\frac{\sum_{i=1}^{n}(\mathrm{Ln(pred)} - \mathrm{Ln(obs)})^2}{n}} \tag{9}$$

in which *n* is the number of observations and

$$\text{bias}\,(\%) = \mathrm{sign}(B)\left[e^{|B|} - 1\right]100 \tag{10}$$

with

$$B = \frac{\sum_{i=1}^{n}\mathrm{Ln(pred)} - \mathrm{Ln(obs)}}{n} \tag{11}$$

and

$$\mathrm{sign}(B) = \begin{cases} 1 \text{ if } B \geq 0 \\ -1 \text{ if } B < 0 \end{cases} \tag{12}$$

RESULTS

The descriptions of the data sets used in this work on the responses of VTEC to environmental conditions relevant to the production and storage of FRMS are shown in Table S1 in the supplemental material. Table S2 in the supplemental material shows the serotypes and strains used for data generation. Despite the high variety in data generation conditions and the large number of serotypes and strains involved, our joint data set showed consistent trends and the models were successfully validated with new observations and independent data from literature and public resources.

Model development. (i) Modeling the growth rate. Figure S1 in the supplemental material shows the comparison between the predicted and the observed growth rates used to fit the model under different conditions of temperature, pH, and a_w. The core model developed with these three environmental factors was extended with a term for the effect of the concentration of undissociated lactic acid which was fitted to the data generated in this study. Figure S1 shows the comparison between the predictions and the observations generated with several concentrations of lactic acid used to extend the model. Table 2 shows the values of the estimated model parameters. As previously reported for *Listeria monocytogenes* (48), we also observed for *E. coli* that the inhibition of the natural logarithm of the growth rate is proportional to the concentration of undissociated acid lactic regardless of pH or total lactic acid concentration (data not shown).

(ii) Modeling the probability of growth. The model describing the dependence of the probability of growth on temperature, pH, and a_w was fitted by logistic regression. A backward coefficient selection procedure indicated that 5 out of the 10 initial model terms were not significant and could be omitted from the model. The model coefficients are shown in Table 2. This model was extended with a term for the effect of the concentration of undissociated lactic acid as fourth environmental factor. Figure S2 in the supplemental material shows the predictions of the model for the probability of growth of VTEC according to three environmental factors—temperature, pH, and a_w—and the predictions of this model extended with the term for undissociated lactic acid. The predicted boundary conditions under which the probability of growth is equal to 0.5 changed significantly when lactic acid was added (see Fig. S2 in the supplemental material). The growth/no-growth boundary pH was close to 4 in the absence of lactic acid, while it reached values of up to 6 when 45,000 ppm of total lactic acid was added into the medium.

(iii) Modeling survival/nonthermal inactivation rates. Survival or nonthermal inactivation refers in this context to the decay of the population under environments characteristic to FRMS in which other factors distinctive from the temperature prevent the growth of *E. coli*. Figure 1A shows model predictions according to the temperature together with the inactivation rates used to fit the model. Temperatures varied from 2 to 37°C and therefore included no-growth temperatures and a wide temperature range at which the growth of *E. coli* is possible. The core model developed to study the dependence of the population decay rate on temperature, pH, and a_w showed that only the temperature exerted a significant effect on the inactivation rate of *E. coli* (Fig. 1A to C).

TABLE 2 Estimates and standard errors for the coefficients of the models and error of the fit

Model, modeled quantity, and equation	Coefficient or other parameter	Estimate	SE
Maximum specific growth rate (μ_{max}), ln(μ_{max}), equation 4	a_0	−9.95	1.25
	a_1	0.188	0.0584
	a_2	1.89	0.253
	a_3	−3.65	4.22
	a_{12}	0.0290	0.00789
	a_{13}	−0.279	0.0955
	a_{23}	4.55	0.572
	a_{11}	−0.00514	0.000295
	a_{22}	−0.223	0.0184
	a_{33}	−131	8.81
	a_4	−0.146	0.0167
	RMSE[a]	0.307	
Nonthermal specific inactivation rate (φ), ln(φ), equation 5	b_0	23.4	1.96
	b_1	−8000	567
	F	−1.51	
	b_4	0.00796	0.000737
	RMSE	0.693	
Thermal specific inactivation rate (τ), ln(τ), equation 6	c_0	−20.3	1.61
	c_1	0.391	0.0281
	RMSE	0.267	
Probability of growth (P), ln(P/1 − P), equation 7	d_0	21.3	1.26
	d_3	7.38	0.468
	d_{12}	0.642	0.157
	d_{13}	−0.583	0.0521
	d_{23}	−0.496	0.121
	d_4	16.5	
	Concordance[b]	97.2%	
	AIC[c]	660	

[a] RMSE, root mean square error.
[b] Concordance between predicted probabilities and observed responses.
[c] AIC, Akaike information criterion.

Thus, when other factors, such as low pH and/or low a_w, inhibited the growth of *E. coli*, the rate of inactivation was dependent exclusively on the temperature, which was not lethal in itself, while it did not depend on the environmental factors causing the growth inhibition and/or inactivation (Fig. 1B and C).

In order to study the effect of the concentration of undissociated lactic acid on the nonthermal inactivation of *E. coli*, a new data set comprising 96 survival curves with several concentrations of lactic acid under various combinations of temperature, pH, and a_w was generated with two *E. coli* serotypes, O157:H7 and O103:H25. The comparison of the inactivation rates between these two serotypes is shown in Fig. 1D. The decay rates of *E. coli* O103:H25 were significantly higher than those of *E. coli* O157:H7 (P value $<$ 0.0001). *E. coli* O157:H7 was more resistant to inactivation, mainly at low pH values. The nonthermal inactivation model predicted faster inactivation rates than those observed with this strain, mainly under the most stringent conditions. The model was modified in order to predict VTEC survival according to the most resistant strain. To do this, a factor, F, calculated from the bias between model predictions and the observed inactivation rates in *E. coli* O157:H7 was added to the model. The data generated with this strain were also used to extend the model for nonthermal inactivation of VTEC with a term for the lactic acid effect. Figure 1E shows the comparison between the predictions and the observed inactivation rates in *E. coli* O157:H7. The model coefficients for the dependence of the nonthermal decay rate on temperature and lactic acid are shown in Table 2.

(iv) Modeling thermal inactivation rate. At temperatures greater than approximately 47°C, the temperature itself exhibits a lethal effect on *E. coli* and there is a sharp change of the effect of the temperature on the inactivation of *E. coli*, which requires the development of a thermal inactivation model (35). Therefore, a thermal inactivation model was developed to describe the lethal effect of temperatures on *E. coli*. To do this, 38 thermal inactivation rates measured at temperatures between 49°C and 55°C and at pH 7 and reported in ComBase were compared with those reported for salami at the same temperature and pH (36, 52). In the latter studies, the response of *E. coli* O157:H7 to several heat treatments, between 49 and 62°C, was quantified in pepperoni at pH 4.4 and 4.8. The greatest heat resistance was observed in salami (36, 52) at pH 4.8 (see Fig. S3 in the supplemental material). This data set was used to fit the thermal inactivation model parameters (Table 2).

Model validation. (i) Validation of the model for the growth rate. The predictions of our model were compared with the predictions of the model for the growth rate of *E. coli* developed by Ross et al. (20) (see Fig. S4 in the supplemental material). The model of Ross et al. (20) predicted, on average, 50% higher growth rates. Ross et al. (20) already warned of possible systematic differences when comparing predictions and observations by other workers attributable to data generation methodology and to the way of estimation of the growth rates. Systematic differences can be quantified by analyzing the two components of the error as previously described when measuring the differences between predictions and observations in foods (53). These two components of the error are the bias and the variability or error of the unbiased parameter or model. These concepts are applicable to the analysis of the overall discrepancy between two models. The estimations of bias and discrepancy percentages between two models can be carried out as previously described (51). A constant bias throughout all the region of comparison means that one of the models gives, on average, greater or smaller predictions by a factor or systematic difference. Disregarding this systematic difference by centering predictions from both models around the same expected value, the discrepancy between our model and the model of Ross et al. (20) is ca. 25%, which is similar to the original error percentage observed between predictions and observations used to fit the models.

Predicted growth rates were also compared with observations in several food types collected from ComBase (see Table S3 and Fig. S4 in the supplemental material). On average, the model was unbiased and the discrepancy between observations and predictions was ca. 120%. Values of 50% for the discrepancy between model predictions and food observations have been reported when analyzing the sources of error in laboratory control experiments (54). However, larger bias factors and discrepancies are commonly found when comparing predictions and observations in complex foods, which comprise numerous factors that affect bacterial growth but are not included in the model, such as the natural food microbiota, food ingredients and preservatives, heterogeneous structure, etc. (55). For instance, errors of 300 to 400% have been estimated between the growth rates of *L. monocytogenes* predicted by four models and those observed in seafood

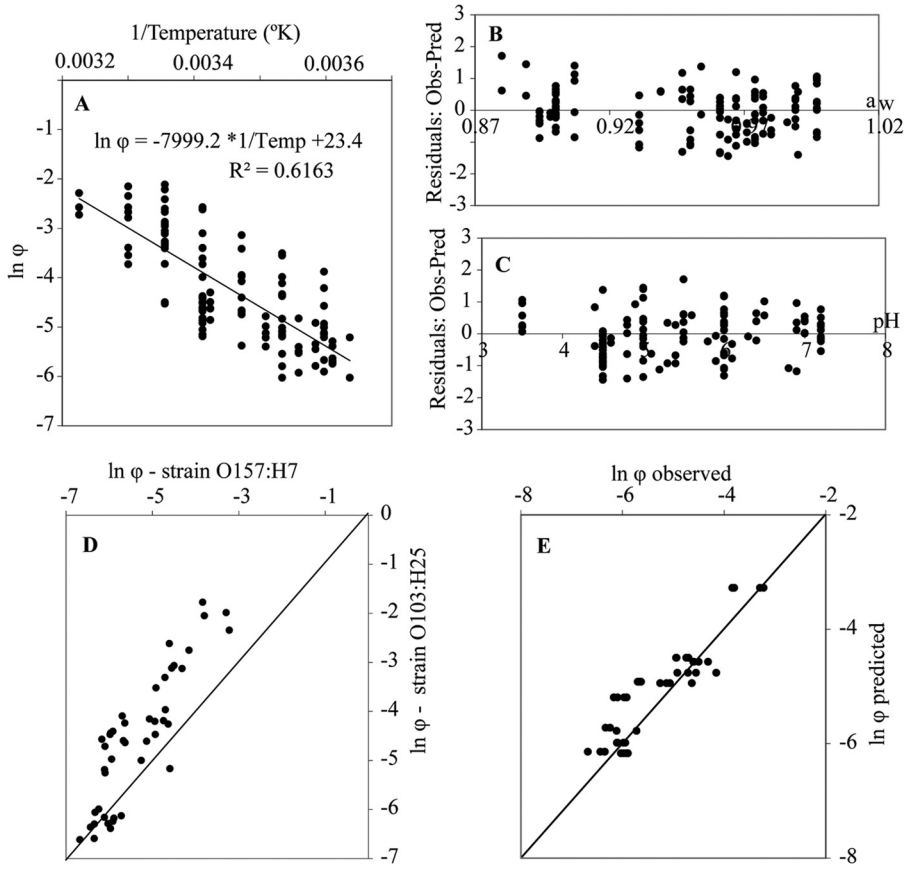

FIG 1 Analysis of the nonthermal inactivation rate of *E. coli* as a function of temperature, pH, a_w, and lactic acid. (A) Observed inactivation rates and predictions by a model that considers only the effect of the temperature (one-factor model). (B and C) The effects of pH and a_w on the inactivation rates was not significant, as shown in the respective residuals plots of the model. (D) Comparison of *E. coli* O157:H7 and *E. coli* O103:H25 nonthermal inactivation rates. (E) Comparison between the observed rates in *E. coli* O157:H7 and the predictions by the one-factor model extended with the effect of the undissociated lactic acid and corrected according to the greater survival of this strain.

inoculated and naturally contaminated with *L. monocytogenes* (56). In a different study, a discrepancy greater than 200% has been reported between rates predicted by broth-based aerobic growth models and observations in ground beef inoculated with *E. coli* (25).

(ii) Validation of the model for probability of growth. Growth and nonthermal inactivation rates obtained from ComBase were coded as 1 and 0, respectively, and used to validate the predicted growth/no-growth boundaries. Figure S5 in the supplemental material shows a good agreement between the predicted boundary conditions, under which the probability of growth is equal to 0.5, and this data set. However, the ComBase data set did not include measurements obtained in the presence of lactic acid.

New data sets were generated in this study in order to explore the performance of the model for the probability of growth under several lactic acid concentrations at various combinations of temperature, pH, and a_w. Figure 2 shows the predicted boundary conditions with and without lactic acid and independent previously published (37, 38) as well as newly generated growth/no-growth data. In general, the predictions of the model extended with a term for the effect of lactic acid were in good agreement with the independently generated growth/no-growth data. The percentage of concordance, estimated as the sum of the number of conditions under which growth was observed and the predicted probability of growth was greater than 0.5 and the number of conditions under which growth was not detected and the predictive probability of growth was smaller than 0.5, was equal to 86% for the extended model including lactic acid, while it was equal to 77% for the original model without the term for the lactic acid (Fig. 2).

(iii) Validation of the survival/nonthermal inactivation rate model. The nonthermal inactivation rate model developed in the present work shows a deviation from the model of McQuestin et al. (35). This is due to the correction factor applied to the model developed in this study in order to predict nonthermal inactivation according to the most resistant *E coli* strain studied in our work (Fig. 1D). Figure S6 in the supplemental material shows the comparison between predicted inactivation rates given by both models from 2 to 37°C. Mainly at high temperatures, the model developed in this study predicts inactivation rates up to 40% lower than the rates predicted by the model of McQuestin et al. (35). A similar discrepancy between the predictions of McQuestin et al. (35) and the observed nonthermal inactivation rates was reported in a previously published study using the same VTEC strains as in our work (38).

(iv) Validation of the model for the concentration of *E. coli* organisms in VTEC-contaminated FRMS manufactured in a pilot plant. The concentration of VTEC organisms predicted as a function of the four environmental factors—temperature, pH, a_w, and lactic acid—was compared with the observed concentration

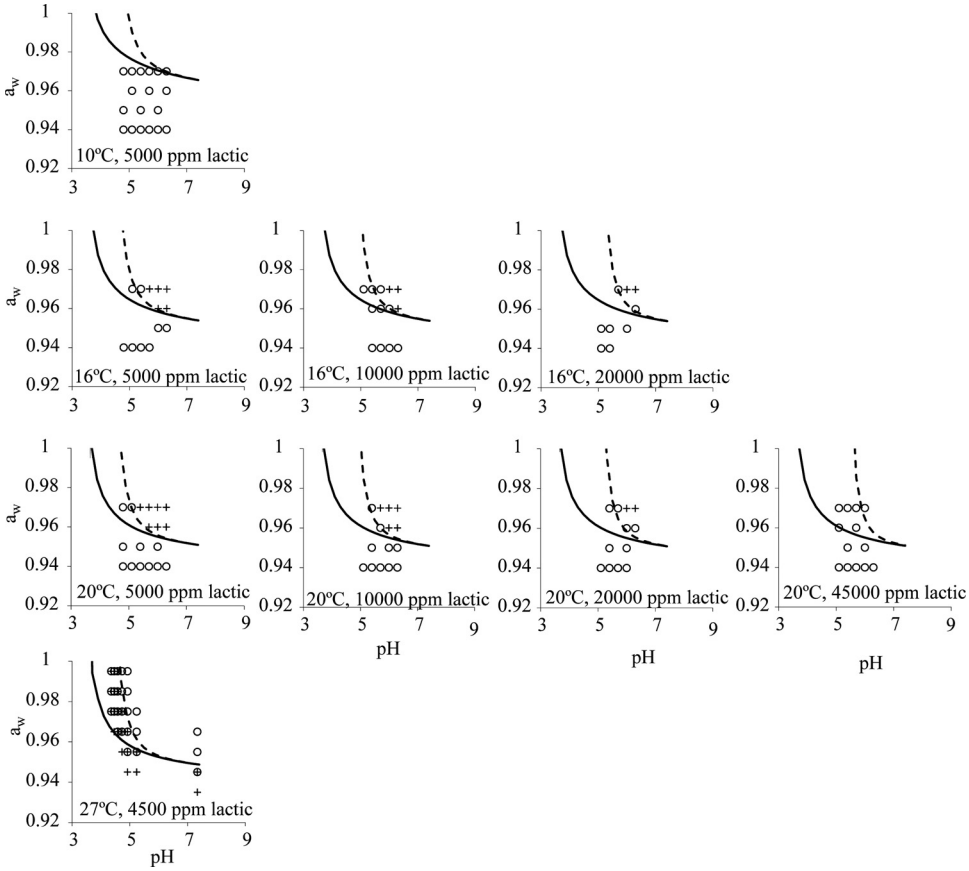

FIG 2 Comparison of the predicted growth/no-growth boundary conditions for VTEC in the presence of lactic acid and independent growth/no-growth data newly generated in this work (upper 8 plots) and previously published (37, 38) (lower plot) with data obtained at 27°C with 4,500 ppm of total lactic acid. The continuous line shows conditions at which the probability of growth is 0.5 as predicted by the three-factor model without considering the effect of the lactic acid. The discontinuous line shows the boundary prediction by the model extended with lactic acid. Symbols denote the observed growth (+) and no-growth (○) conditions independently generated in our study.

during the manufacturing and storage of VTEC contaminated Norwegian sausage (Fig. 3A) and Swedish mettwurst (Fig. 3B) in a pilot plant. The fermentation took place at ca. 24 and 22°C, respectively (Table 1). No significant differences in the inactivation of *E. coli* were observed between these two products (Table 1).

Two batches of Norwegian sausage, initially contaminated with a cocktail of the strains O157:H7 218Rif and O103:H25 Rif^r, were fermented at 24 and 34°C and stored at 12, 16, 21, and 25°C for ca. 66 days (Table 1 and Fig. 3C to E). The models implemented in EcSF predicted slight differences in the reduction of VTEC during fermentation, with greater reductions at higher fermentation temperatures (Table 1). However, these differences were small and could not be observed in the data measured in the manufactured sausages because of the measurement error inherent to food plate counts (Table 1). In general, during the 27 days of fermentation plus maturation, VTEC concentration decreased by ca. 1 decimal log, regardless of the temperature of fermentation. The greatest reduction of VTEC was observed during storage at high temperatures (Table 1). At 25°C, VTEC was not detected after 66 days of storage (Fig. 3D and F and Table 1). At 21°C, reductions of 3 to 4 decimal logarithmic units were observed during the storage period, while decreases of less than 2 decimal logarithmic units were observed after 66 days of storage either at 16°C or at 12°C (Table 1 and Fig. 3C and E). The predicted concentrations were in good agreement with the observations for all VTEC curves throughout FRMS elaboration and storage, showing discrepancy percentages between 3 and 16% (Table 1).

(v) Comparison of the model performance throughout FRMS production and storage with other modeling approaches. For further validation, we compared the predicted concentrations by our modeling tool, EcSF, with the approaches of Ross et al. (20) and Mellefont et al. (19) (Meat & Livestock Australia refrigeration index calculator [MLA-RIC]) and Ross and Shadbolt (34) (Meat & Livestock Australia *E. coli* inactivation in fermented meat model [MLA-EcIFM]). The EcFS simulates the solution for equation 1 to predict the concentration of VTEC under environments fluctuating between growth, survival, and thermal inactivation conditions characteristic to FRMS manufacturing and storage. The MLA-RIC predicts the refrigeration index, which is the decimal logarithmic increase in the concentration of *E. coli* in meat at a given time-temperature profile. The MLA-EcIFM model predicts the inactivation of *E. coli* in uncooked, comminute, fermented meat products or analogous environments in which inactivation conditions are determined by low a_w and/or pH, at a given time-temperature profile.

Three flow diagrams in Fig. 4 describe meat processing (F1), FRMS elaboration (F2), and the concatenation of both operations

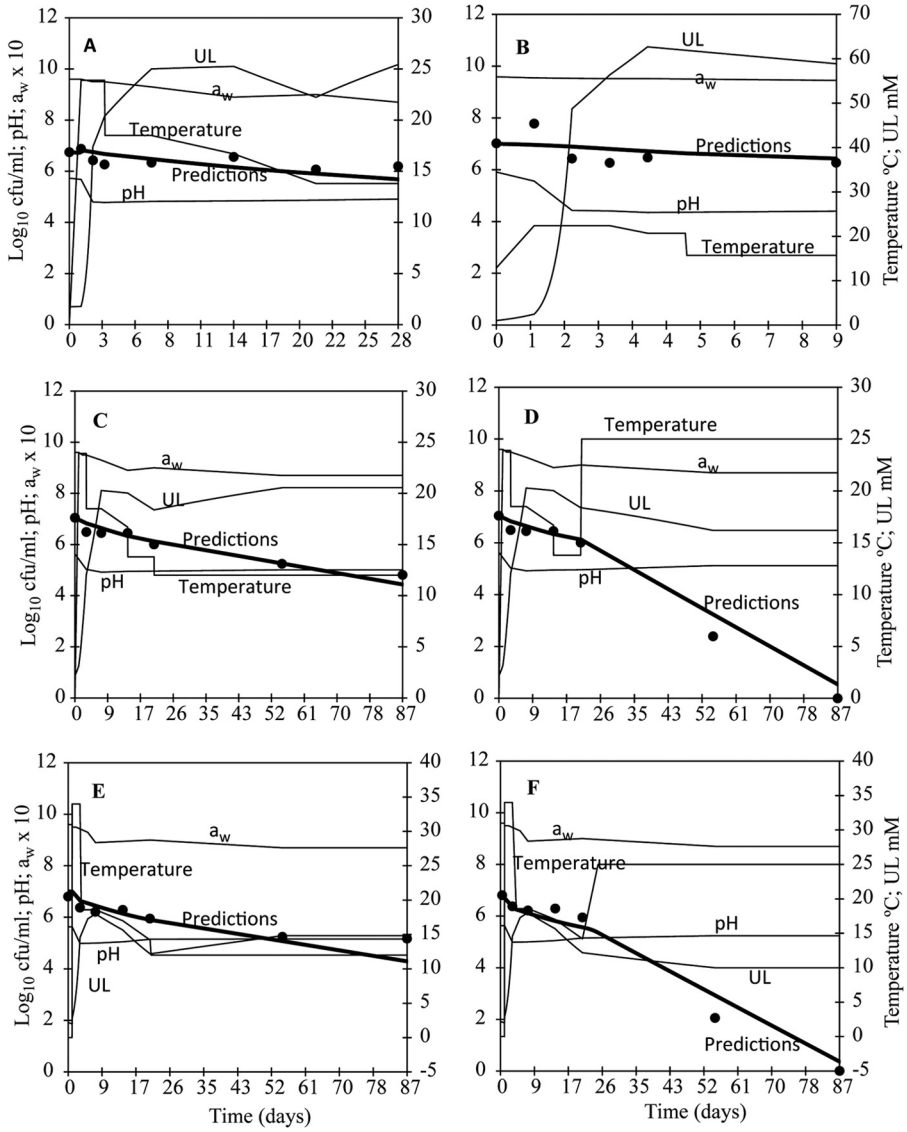

FIG 3 Predictions and observations of VTEC during the elaboration process of Norwegian sausages (A) and Swedish mettwurst (B) fermented at 24 and 22°C, respectively, and during elaboration and storage of Norwegian sausages fermented at 24°C and storage for 66 days at 12°C (C) or at 25°C (D) and fermented at 34°C and stored for 66 days at 12°C (E) or at 25°C (F).

in a continuous process (F1 plus F2). Figure 4 shows times and conditions for each phase in each diagram.

The processing of meat (F1) starts after the slaughter process of the animal in the slaughterhouse and the postmortem inspection by the competent authority. Meat must be stored and manipulated in accordance with the requirements laid down in reference 57. Two scenarios were taken into account (Fig. 4): the standard process with immediate refrigeration of the carcass (scenario A) and the hot boning and cutting alternative (scenario B). These processing scenarios were combined with two production options: minced meat and meat preparations produced from animals other than poultry 6 days after processing and meat preparations produced with boned, vacuum-packed beef and veal meats 15 days after processing. The response of VTEC was simulated with the MLA-RIC and the EcSF in all scenarios and production options. For scenario A, with immediate refrigeration of the carcass previous to the boning and cutting operation, the EcSF predicted an initial increase of 0.5 log_{10} CFU/g of VTEC in the first 2.5 h, followed by a decrease of 0.10 log_{10} CFU/g of VTEC in meat preparations produced 6 days after processing, and the same initial increase followed by a reduction of 0.22 log_{10} CFU/g in meat prepared 15 days after processing. For the hot boning and cutting alternative, or scenario B, the EcSF predicted an increase of 0.91 log_{10} CFU/g of VTEC in the first 3.5 h, followed by the same predicted reductions as in scenario A. The MLA-RIC considers only growth and predicted similar increases, 0.73 and 1.03 log_{10} CFU/g, of VTEC in scenarios A and B, respectively.

The VTEC concentration throughout FRMS manufacturing according to flow F2 (Fig. 4) was predicted with the MLA-EcIFM and the EcSF. Two general types of FRMS were considered for the comparison: dry and semidry (34, 58). Two possible initial meat temperatures were taken into account for the production of FRMS

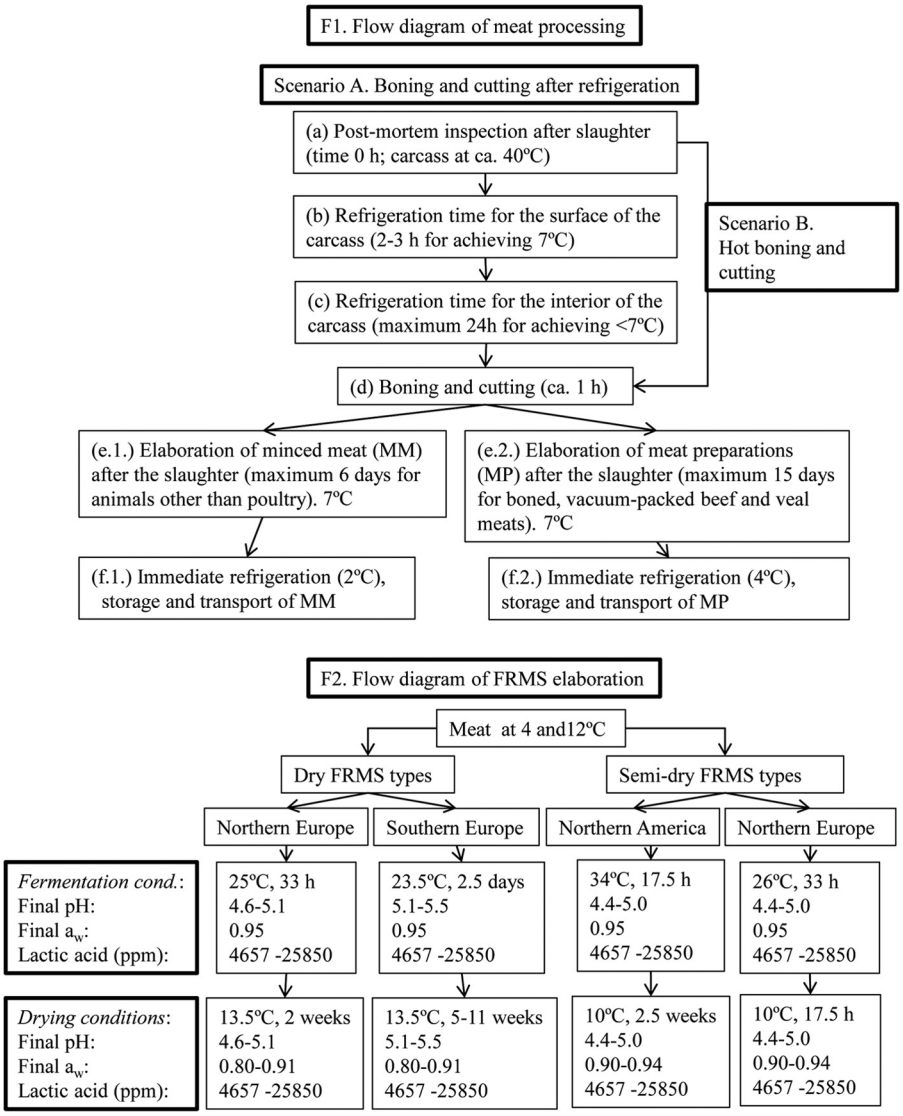

FIG 4 Flow diagram of meat processing (F1) and of manufacturing several types of FRMS (F2). In F1, the a_w in meat was assumed to be equal to 0.995 and the pH of the meat equal to 5.8. Temperature refers to surface temperature in carcasses and meat pieces. *cond.*, conditions.

(Fig. 4 and Table 3). These temperatures were the standard refrigeration temperature for meat preparations, 4°C, and the maximum permitted room temperature in the slaughterhouse for boning and cutting, 12°C. Due to the wide variability of temperatures, pHs, and a_ws, lactic acid concentrations, and time intervals used in FRMS production, average values were used as input environmental conditions (Fig. 4 and Table 3).

Both approaches, MLA-EcIFM and EcSF, predicted the smallest VTEC reduction, i.e., smaller than 1 decimal logarithmic unit, in the Northern Europe semidry FRMS because of the short drying step (17.5 h). Greater reductions were predicted in dry FRMS and the Northern America semidry variety (Table 3). According to the predictions of both approaches, the safest product, i.e., with the greatest reduction of VTEC concentration, would be the traditional dry Southern Europe type due to a long drying period (11 weeks) and thus a longer exposure of VTEC to inactivation conditions. The EcSF tool predicted, in general, lower reductions of VTEC concentration than the MLA-EcIFM approach. This difference was due to the correction of the nonthermal inactivation model implemented in the EcSF with a factor calculated from the inactivation rates generated in this study with the most resistant strain, which was *E. coli* O157:H7 (Fig. 1D).

Predictions on VTEC concentration were obtained at the unified flows, F1 plus F2, representing the entire process from animal slaughter to the final product using the EcSF tool. The predicted reduction of VTEC concentration throughout these processes was lower than those observed alone in flow F2 because of the increase in the concentration predicted during meat processing in flow F1 (Table 3). As predicted in the flow for FRMS manufacturing only, inactivation of VTEC was greater in general in dry than in semidry fermented sausages, and the greatest inactivation was predicted in the dry Southern Europe FRMS type traditionally elaborated (Table 3). An increase of VTEC concentration was predicted in all cases for the Northern Europe semidry variety as well as in other FMRS types manufactured after hot boning and cutting (Table 3).

These scenarios for FRMS production were simulated to illus-

TABLE 3 Predicted reduction of VTEC concentration by MLA-EcIFM and EcSF during manufacturing of several types of FRMS according to the production flows described in Fig. 4

	Predicted reduction of VTEC (log_{10} CFU/g) in flow[a] at indicated initial temp[b] and with indicated processing of meat[c]							
	F1							
	MLA-EcIFM		EcSF[d]		F1 + F2, EcSF[e]			
FRMS type	4°C	12°C	4°C	12°C	Cold, 6 days, 2°C	Cold, 15 days, 4°C	Hot, 6 days, 2°C	Hot, 15 days, 4°C
Dry								
Northern Europe	1.46	1.46	0.46	0.43	0.13	0.25	Δ0.29[f]	Δ0.17
Southern Europe	3.29	3.29	1.05	1.01	0.69	0.81	0.27	0.39
Southern Europe (traditional)	6.55	6.55	2.13	2.1	1.92	2.04	1.50	1.62
Semidry								
Northern America	1.41	1.41	0.43	0.37	0.1	0.22	Δ0.32	Δ0.2
Northern Europe	0.45	0.45	0.11	0.07	Δ0.3	Δ0.18	Δ0.72	Δ0.6

[a] See flow diagrams in Fig. 4.
[b] Possible scenarios for meat processing according to Fig. 4, cold and hot cutting and boning and 6 and 15 days at 7°C before preparation.
[c] Initial temperatures of the meat used in FRMS manufacturing according to Fig. 4.
[d] In F2 simulation with EcSF, the final value for lactic acid is 4,657 ppm as reported in MLA- EcIFM documentation; a_w was assumed to decrease at a rate of 0.014 units per day during the drying step.
[e] In F1 + F2 simulation with EcSF, the final concentration of lactic acid is the average final value observed in the sausages manufactured in the pilot plant in this work, i.e., 25,850 ppm.
[f] Δ, increase in concentration.

trate the potential use of the prediction tools available for VTEC, although some of the specific conditions used in the simulations may not reflect real manufacturing environments.

DISCUSSION

Kinetic and probability models have been integrated to give dynamic predictions for environments that fluctuate in a range of growth, survival, and inactivation conditions. Predictions for the concentration of VTEC throughout FRMS manufacturing and storage have been compared with independent observations. The model is implemented in the EcSF computing tool, which predicts the concentration of VTEC during FRMS elaboration and allows the estimation of storage conditions and manufacturing steps required to reach a given reduction in the concentration of VTEC.

Predictions when models are empirical as in this work are reliable only if inside the interpolation region of the model. The interpolation region of the model was defined in a landmark paper as the minimum convex region containing all the environmental conditions under which measurements used to fit the model were obtained (59). The interpolation region of the model is different from the nominal region or product of the ranges of the environmental variables where observations were obtained (59). These ranges are described in Table S1 in the supplemental material for our models. The interpolation region of the EcSF tool was estimated using the DMFit tool (60) from the nonthermal inactivation, thermal inactivation, and growth data sets used to fit the population kinetic models, and its vertices are reported in Table S4 in the supplemental material. The interpolation region is inside the model nominal region but generally smaller; the percentage of overlap between both regions can be estimated by Monte Carlo methods as previously described (61). We have estimated that the interpolation region of the EcSF tool is 30% of its nominal region. Therefore, there is a high risk of extrapolation if conditions are chosen randomly inside the nominal region. This is of high importance because the error of model predictions obtained under environmental conditions lying outside the interpolation region of the model is increasingly greater as the distance to the edge of the interpolation region increases (53). While large variations in FRMS manufacturing conditions are not expected and therefore are likely to lie within the interpolation region of the EcSF tool, it is recommended to check that the vertices of the set of conditions forming the dynamic environmental profile are inside the interpolation region of the model.

We found that when factors distinctive from the temperature, such as low pH and/or low a_w, inhibited the growth of *E. coli*, the rate of inactivation was dependent on the temperature and, to a smaller degree, also on the undissociated lactic acid, which were not lethal in themselves, while it did not depend on the environmental factors causing the growth inhibition and/or inactivation, i.e., pH and/or a_w. Similar results have been previously reported for *E. coli* (35, 62). We observed that the rates of inactivation of *E. coli* increased as the temperature increased from 2 to 37°C when the values of pH and/or a_w were growth inhibitory. The increase of the rates could be due to the acceleration of the inactivation cellular processes as the temperature increases in this range. For most bacteria, enzymatic rates increase proportionally to the temperature in the range of 20 to 40°C. Another explanation for the greater sensitivity of *E. coli* to inactivating pH and/or a_w values at high temperatures could be the change in the properties of the membrane. Bacterial acid tolerance mechanisms are associated with membrane composition, such as lipid content that changes proton permeability (63). Acid tolerance is also based on the effectiveness of ion transport through the cell membrane to maintain a constant internal pH (64) and on the induction of proteins responsible for repairing the membrane (65). On the other hand, the mechanism responsible for the death of *E. coli* during exposure to increasing osmotic pressure has been reported to be a combination of membrane deformation and structural changes of the membrane lipids affecting permeability (66). Therefore, perturbations of the membrane properties associated with relatively high temperatures could be one of the factors causing the decrease of *E. coli* survival at inactivating pH and/or a_w values. It has been ob-

served that as temperature increases there is an increase in saturated fatty acids together with a decrease in unsaturated fatty acids of *E. coli* cell membranes, affecting their consistency (67–69). Heat shocks at 42°C have been reported to destabilize the membrane, changing the phospholipids and fatty acid profiles and increasing its permeability (32, 69, 70). Other researchers (71, 72) indicated that exposure to 42°C decreased the membrane anisotropy and increased its rigidity.

In this study, we have observed that the concentration of undissociated lactic acid affected growth and inactivation rates and had a considerable impact on the growth/no-growth boundary conditions (see Fig. S2 in the supplemental material). Organic acids are widely used preservatives in the food industry (73). Despite this use, their antimicrobial mode of action is still not fully understood (74). It is generally agreed that the ability of weak acids to inhibit microbial growth is related to their membrane permeability. At pH values lower than the pK_a of their acidic group, the acid is majorly uncharged, and in this form the acid can pass freely through the cell membrane. At higher pH values the acid is mainly in dissociated or charged form; the transport of the dissociated acid through the membrane cannot take place by free diffusion but requires a less efficient secondary transport mechanism (75). Early studies suggested that effects on intracellular pH were key to understanding the toxicity of weak acids. Inside the cell, lactic acid dissociates, releasing protons that decrease the internal pH and anions that in addition specifically inhibit different aspects of metabolism and can have osmotic effects on the cell resulting in impaired growth (74, 76–78). Available evidence indicates that it can also affect membrane function (74). Undissociated lactic acid has been shown to disrupt the lipopolysaccharide layer of the outer membrane in *E. coli*, increasing its permeability (79–81). These actions on the cell are likely to contribute to the growth-inhibitory effects of other environmental factors and to narrow the growth environmental region.

The EcSF predictive tool can be applied for the evaluation of the impact of modifications, interventions, or unexpected events during the manufacturing process and/or storage period on VTEC survival. As an example, EcSF predicts that the raising of the fermentation temperature from 20 to 28°C results in a ca. 100% increase of VTEC inactivation. Similarly, the impact of changes during maturation or storage can be evaluated under the exact conditions for each particular FRMS manufacturing process. Thus, the EcSF program can be used to optimize FRMS production in order to achieve the required reduction in the concentration of *E. coli* set by the relevant food safety authorities (11, 12). The models implemented in EcFS are based in the most resistant strains of *E. coli* found in our study, and therefore, EcSF is likely to overpredict VTEC concentration in FRMS. The design of an optimum FRMS manufacturing and storage process to achieve a required reduction of VTEC and the current efforts directed toward minimizing the prevalence of VTEC in raw meat are approaches able to enhance the safety of these meat products regarding VTEC.

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